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Induction of ethylene biosynthesis and necrosis in weed leaves by a *Fusarium oxysporum* protein

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A small assortment of microbial proteins have the ability to activate defense responses and induce necrosis in plant cells through cell signaling pathways. These proteins are of interest because of their potential use as bioherbicides and inducers of plant resistance in agriculture. A 24-kDa protein (Nep1) was purified from culture filtrates of *Fusarium oxysporum,* and the effects of this protein on weed leaves were investigated. This protein induced necrosis in detached leaves of *Papaver somniferum, Lycopersicon esculentum, Malva neglecta,* and *Acroptilon repens* when taken up through the petiole. The pattern and level of necrosis were dependent on the plant species. Treatment with Nep1 induced the production of ethylene in isolated leaves of various species, and the level of ethylene response was shown to be correlated to the concentration of the protein. Pretreating leaves of *P. somniferum, L. esculentum, M. neglecta,* and *Cardaria draba* with 100 μl L^{-1} ethylene enhanced the protein induction of ethylene biosynthesis in those leaves. Application of Nep1 (200 nM) as a spray to intact plants of *Abutilon theophrasti, P. somniferum, Centaurea solstitialis, Centaurea maculosa,* and *Sonchus oleraceus* resulted in extensive necrosis of leaves within 48 h. The results of this research are supplemental to our understanding of the role of specific polypeptides in plant/microbe interactions and demonstrates for the first time that a fungal protein can cause extensive necrosis when applied to weed species as a foliar spray.

Nomenclature: *Fusarium oxysporum* Schlechtend:Fr. f. sp. *erythroxyli; Malva neglecta* Wallr., MALNE, common mallow; *Cardaria draba* L., CADDR, hoary cress; *Papaver somniferum* L., PAPSO, opium poppy; *Acroptilon repens* L. CENRE, Russian knapweed; *Centaurea maculosa* Lam., CENMA, spotted knapweed; *Sonchus oleraceus* L. SONOL, annual sowthistle; *Abutilon theophrasti* Medicus, ABUTH, velvetleaf; *Centaurea solstitialis* L. CENSO, yellow starthistle; *Lycopersicon esculentum* L. 'Bonnie Best,' tomato.

Key words: Bioherbicide; elicitor; ethylene; Silwet L-77; hairy vetch.

There are polypeptides produced by plant pathogens and saprophytic organisms that induce defense responses and necrosis in plant tissue (Anderson et al. 1993; Bailey 1995; De Wit and Spikman 1982; Ricci et al. 1993; Sutherland and Pegg 1995; Wei et al. 1992; Yu 1995). For most of these polypeptides, however, their roles in specific plant/pathogen interactions are not fully understood. The 24-kDa protein (Nep1) from *Fusarium oxysporum* Schlechtend.Fr., which causes necrosis in dicot plant species, is being considered as a bioherbicide for weed control. At the same time, a bacterial protein with similar activities, harpin, is being evaluated for use as an inducer of plant disease defense mechanisms (Bauer et al. 1997; Dong et al. 1997; Qui et al. 1997; Theisen et al. 1997). Considering these contrasting approaches to the use of microbial polypeptides, it is important to determine the response of various weed species to these compounds.

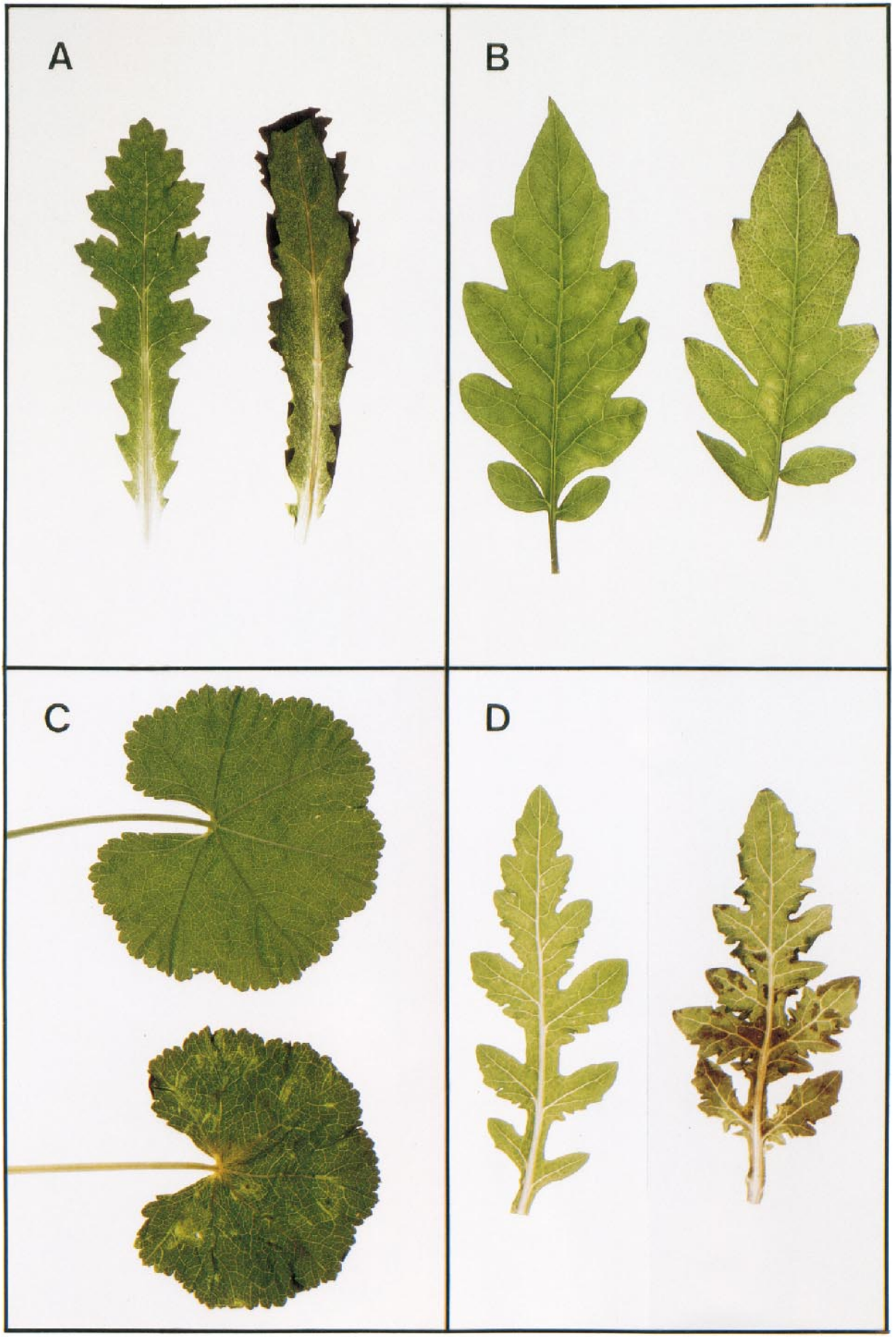
Nep1 elicits physiological responses in a broad range of plant species (Bailey 1995). *F. oxysporum* is of particular interest because several isolates of this fungus are currently being evaluated as biocontrol agents for narcotic plants, such as *Papaver somniferum* and *Erythroxylum coca* (coca) and *E. novogranatensis* (coca) (Bailey et al. 1997a; Fravel et al. 1996; Hebbar et al. 1996), and for various weeds (Boyette et al. 1993; Kremer and Schulte 1989; Pandey et al. 1992). Most of the isolates of *F. oxysporum* that have been examined produce Nep1, though the role of the protein in disease development or induced resistance, if any, remains unknown.

Necrosis is one of the visible responses of leaves to Nep1 (Bailey 1995). Another response that can be measured is the production of the plant hormone ethylene. Ethylene biosynthesis is a characteristic response of plant cells to certain microbial proteins (Anderson et al. 1993, 1997; Bailey et al. 1997b; Felix et al. 1991; Fuchs et al. 1989; Hammond-Kosack et al. 1996; Yu 1995), and ethylene is a critical hormone involved in the mediation of stress responses, including disease development (Abeles et al. 1992; Boller 1991). The goal of this work was to determine the response of weed species to Nep1.

Materials and Methods

Purification of Nep1

The isolates of *F. oxysporum* Schlechtend:Fr. used in these studies were, CP3A, a pathogen of *P. somniferum* and EN-4, a pathogen of *E. coca* and *E. novogranatensis* (Bailey et al., 1997b). One-liter cultures were grown for 7 d in Czapek-Dox broth with 1% casamino acids at 25 C and 150 rpm. Nep1 was purified from culture filtrates as previously described (Bailey 1995) using ultrafiltration and Fast Protein Liquid Chromatography.[1] The purity of Nep1 was deter-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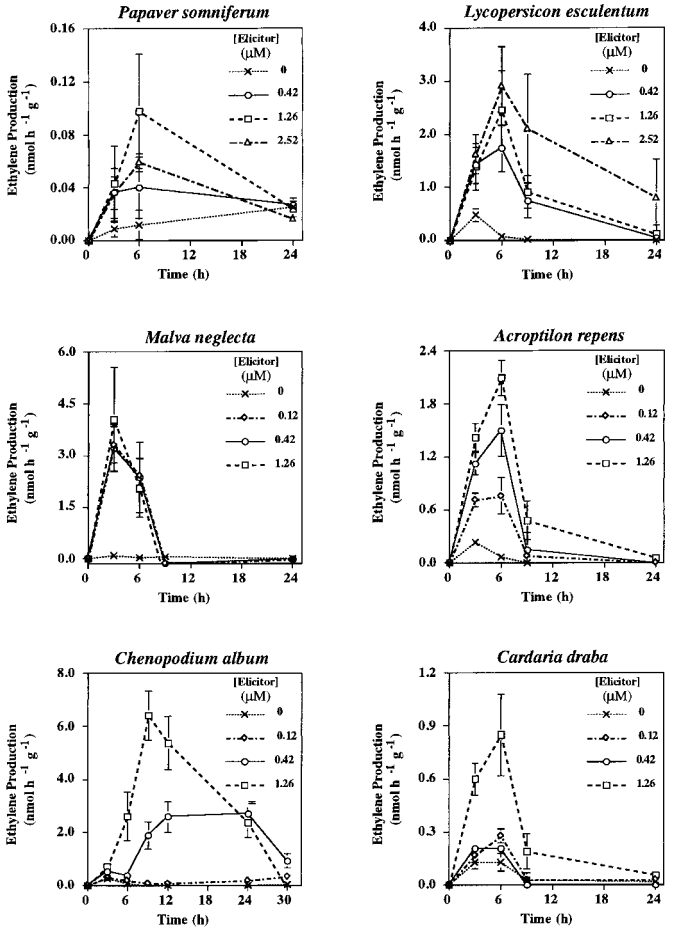

FIGURE 2. Rates of ethylene production by detached leaves treated in 0, 0.12, 0.42, 1.26, or 2.52 μM Nep1 solution. Petioles were placed in the appropriate concentration of Nep1 for 15 min, after which the leaves were moved to a buffer solution for 45 min before transferring to a container containing water. The container was sealed, and air samples were removed with a syringe at appropriate times. Each point is the mean value for eight leaves from two independent experiments, and the error bars are the standard error for each mean.

mined by separating aliquots of each fraction by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Schägger and von Jagow 1987) with silver staining (Wray et al. 1981). Quantification of purified protein was based on the method of Bradford (1976) with bovine serum albumin as the standard. The purified protein was shown to be a single 24-kDa peptide when visualized on a silver-stained gel, and it was recognized by antibodies raised against Nep1 (Bailey 1997c).

Growth of Plants

P. somniferum seeds were planted in Jiffy-Mix Plus[2] in 10-cm pots, and the resulting seedlings were thinned to one plant per pot. Plants were maintained in a growth chamber. The 11:13-h light:dark photoperiod alternated between 28 and 22 C, respectively. Illumination was from fluorescent bulbs that produced 400 mmol m^{-2} s^{-1} at the level of the leaves, and relative humidity was maintained at 40%.

L. esculentum seeds were planted in Jiffy-Mix Plus in 10-cm pots under greenhouse conditions with natural lighting at 26 C. Weed seeds were from the Beltsville Weed Science Laboratory collection and were also grown under greenhouse conditions with natural illumination at 26 C. *M. neglecta, C. solstitialis, C. maculosa, S. oleraceus,* and *A. theophrasti* were grown in Jiffy-Mix Plus, whereas *C. album, A. repens,* and *C. draba* were grown in a mixture of soil, sand, and Jiffy-Mix Plus (3:1:1 by volume).

Leaves of various other weed and crop species were harvested between late August and early October from plants growing in fields at the Beltsville Agricultural Research Center. Leaves were cut from the plant, placed in a plastic bag containing wet paper towels, and brought to the laboratory within 1 h. Once in the laboratory, the leaves were rinsed with deionized water, the petioles were recut and placed in water, and the leaves were used in induction studies.

Protein Treatment and Ethylene Measurement

P. somniferum plants were 6 wk old and *L. esculentum* plants were 8 wk old when their leaves were used in experiments. *M. neglecta, C. album, A. repens,* and *C. draba* plants were several months old when their leaves were used. However, leaves were chosen that were fully expanded and approximately the same size, age, and appearance within any given species. Petioles were placed in buffer (10 mM MES, pH 6.0 with 250 mM sorbitol) containing Nep1 or buffer solution for the controls for 15 min to allow the uptake of protein, then transferred to buffer for an additional 45 min. Each petiole was then placed in water, and the leaf was sealed inside a 60- or 125-ml glass culture tube, depending on its size. Tubes were sealed with rubber septa or rubber stoppers equipped with septa to allow for the withdrawal of air samples by needle and syringe. At each sampling time, 3 ml of air were injected into each sealed culture tube with a syringe and needle, the syringe was pumped several times to mix the atmosphere in each tube, and then a 3-ml sample was removed for ethylene quantitation by gas chromatography with flame ionization detection (Hewlett-Packard 5890A Gas Chromatograph).[3] The GC was equipped with a 1-ml injection loop and a 3-m Hayesep D 80/100 separation column heated to 80 C. Helium was the carrier gas, and the minimum level of detection was about 10 nl L^{-1} ethylene in 1 ml. For experiments involving ethylene pretreatment prior to protein treatment, leaves were removed from plants that were pretreated for 16 h in air or 100 μl ethylene L^{-1}. After ethylene pretreatment, the containers were opened; leaves were removed and placed on the laboratory bench for several minutes before they were used.

In addition, whole plants (4–6 wk old) of *C. solstitialis, A. repens, C. maculosa, P. somniferum,* and *S. oleraceus* were treated by direct spray of Nep1 (200 nM, 5 μg ml^{-1}) in a 0.1% Silwet L-77 (1,1,1,3,5,5,5-heptamethyltrisiloxanyl propyl-methoxy-poly[ethylene oxide], CAS registry no.

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FIGURE 1. Effect of Nep1 on detached leaves: (A) *Papaver somniferum,* (B) *Lycopersicon esculentum,* (C) *Malva neglecta,* and (D) *Acroptilon repens.* Solutions were taken up through the vascular system. Control leaves are on the left or top in each set, and Nep1-treated leaves are on the right or bottom. The protein concentrations used for treatment were 2.52, 1.26, 1.26, and 0.42 μM, respectively. Photographs were taken 2 d after treatment.

Table 1. Ethylene production by leaves of various plant species treated with Nep1.

Species (common name)	Ethylene production (nmol g^{-1})	
	Control	Treated
Plantago major L.(broadleaf plantain)	0.5 ± 0.2	1.9 ± 0.3
Plantago lanceolata L.(buckhorn plantain)	1.2 ± 0.1	1.2 ± 0.4
Stellaria media L.(common chickweed)	0.9 ± 0.2	1.4 ± 0.2
Chenopodium album L.(common lambsquarters)	0.6 ± 0.1	60.2 ± 10.4
Malva neglecta	0.5 ± 0.2	4.0 ± 0.7
Phytolacca americana L. (common pokeweed)	0.5 ± 0.3	0.4 ± 0.0
Rumex crispus L.(curly dock)	1.1 ± 0.3	7.3 ± 2.1
Taraxacum officinale L.(dandelion)	0.8 ± 0.3	1.5 ± 0.4
Panicum dichotomiflorum Michx.(fall panicum)	0.6 ± 0.1	1.0 ± 0.2
Vicia sativa (hairy vetch)	1.5 ± 0.2	69.3 ± 16.9
Polygonum pennsylvanicum L.(Pennsylvania smartweed)	0.3 ± 0.1	0.4 ± 0.1
Rorippa teres (Michx.) Stuckey (yellowcress)	1.3 ± 0.1	5.5 ± 1.2
Amaranthus hybridus L.(smooth pigweed)	0.4 ± 0.3	3.3 ± 1.0
Glycine max L.(soybean)	0.9 ± 0.4	0.7 ± 0.2
Abutilon theophrasti Medicus (velvetleaf)	1.5 ± 0.5	2.1 ± 0.5
Morus alba L.(white mulberry)	4.7 ± 0.9	5.8 ± 3.6
Duchesna indica L.(Indian mock-strawberry)	0.5 ± 0.1	3.8 ± 0.9
Setaria glauca L.(yellow foxtail)	0.3 ± 0.0	0.2 ± 0.1
Cyperus esculentus L.(yellow nutsedge)	0.2 ± 0.0	0.3 ± 0.1

[a] Treated leaves were placed in 1.25 μM (30 μg ml^{-1}) purified Nep1 solution for 15 min; then they were transferred to containers containing buffer without Nep1. The containers were sealed, and total ethylene production was measured after 24 h. Each value is the mean for 6 leaves from 2 independent experiments; standard errors are also shown.

27306-78-1).[4] Photographs were taken 7 d after treatment for whole plants and 2 d after treatment for detached leaves.

All experiments were repeated one or more times with at least three plants or leaves per treatment. Standard errors of the mean were determined and error bars plotted on the graphs.

Results and Discussion

Nep1 is one of the primary polypeptides found in culture filtrates of several *Fusarium* species (Bailey et al. 1997c). The function, if any, of this protein in a plant/pathogen interaction is still unknown, but it induces severe necrosis in detached *P. somniferum* leaves when delivered through the vascular system of the leaf (Figure 1A). The pattern of necrosis follows the veins of the leaf, indicating movement of this protein or another soluble signal throughout the vascular tissue. The protein is also capable of inducing necrosis in the leaves of various crop species (Bailey et al. 1997c), including *L. esculentum* (Figure 1B) and the leaves of weeds such as *M. neglecta* and *A. repens* (Figures 1C and 1D). However, the necrotic response is species dependent. In *L. esculentum,* necrosis occurred in localized spots characteristic of a hypersensitive response throughout the leaf surface (Figure 1B). *M. neglecta* leaves responded to Nep1 with a patchy pattern of chlorosis, whereas *A. repens* leaves developed localized regions of severe necrosis. The level of necrosis increased with time after treatment (data not shown). *C. album* and *C. draba* did not respond to protein treatment with visible necrosis at the concentrations of 0.13 to 1.25 μM (data not shown).

P. somniferum, L. esculentum, M. neglecta, A. repens, C. album, and *C. draba* leaves responded to treatment with Nep1 by producing ethylene (Figure 2). Ethylene biosynthesis is rapidly induced in various plant species in response to other microbial proteins (Anderson et al. 1993, 1997; Felix et al. 1991) and represents the activation of at least two enzymes, ACC synthase and ACC oxidase (Avni et al. 1994). Induction of ethylene biosynthesis was species, time, and protein concentration dependent, though the peak in the rate of ethylene production was usually around 6 h after treatment. In general, higher concentrations of Nep1 (up to 1.25–2.5 μM) induced higher levels of ethylene production. The concentration of Nep1 needed to induce the maximum rate of ethylene biosynthesis in *C. album* and *C. draba* leaves is probably closer to the higher level. Variations in the amount of ethylene produced by the different species in response to this protein may be due to the age of plant tissue used and to inherently different abilities of those species to produce and perceive ethylene.

Field-grown plants were also tested for responsiveness to Nep1 (Table 1). Several of the species that were evaluated had little or no response (less than a doubling in ethylene production) when treated with 1.25 μM protein solution. Many species, though, did respond strongly to Nep1 (greater than a tripling in ethylene production), including *P. major, C. album, M. neglecta, R. crispus, V. sativa, R. teres, A. hybridus,* and *D. indica. V. sativa* also showed severe necrosis (data not shown). In a previous study (Bailey 1995), none of the grass species *Zea mays* L. (corn), *Triticum aestivum* (wheat), *Stenotaphrum secundatum* (St. Augustine grass), and *Phalaris arundinacea* (reed canary-grass) responded to Nep1. In this study, *S. glauca* did not respond, but *P. dicotomiflorum* had a small increase in ethylene production in response to Nep1, but there was no leaf necrosis. This selective responsiveness has been observed with another fungal protein, the ethylene-inducing xylanase (EIX) from *Trichoderma viride,* on *Nicotiana tabacum* (tobacco) and *L. esculentum.* Here a single dominant gene confers sensitivity to EIX (Bailey et al. 1993), and the gene appears to be associated with recognition of the EIX protein (Hanania and Avni 1997). This

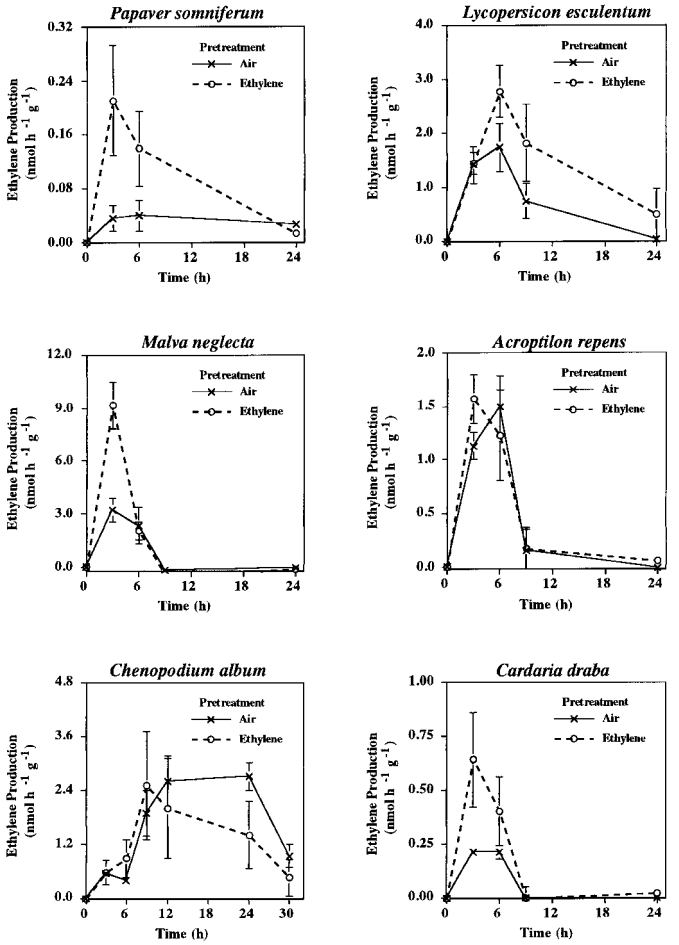


FIGURE 3. Effect of ethylene pretreatment on induced ethylene production. All leaves were treated in 0.42 μM Nep1 solution or a buffer solution for 15 min after pretreatment in air or 100 μl L^{-1} ethylene. After treatment, the leaves were moved to a buffer solution for 45 min before transferring to a container containing water. The container was sealed, and air samples were removed with a syringe at appropriate times. Each point is the mean value for eight leaves from two independent experiments, and the error bars are the standard error for each mean. Buffer-only controls are not shown for clarity because treatments without Nep1 resulted in minimal ethylene production.

would imply that the cell signaling pathways of different plant species are induced by different microbial messengers.

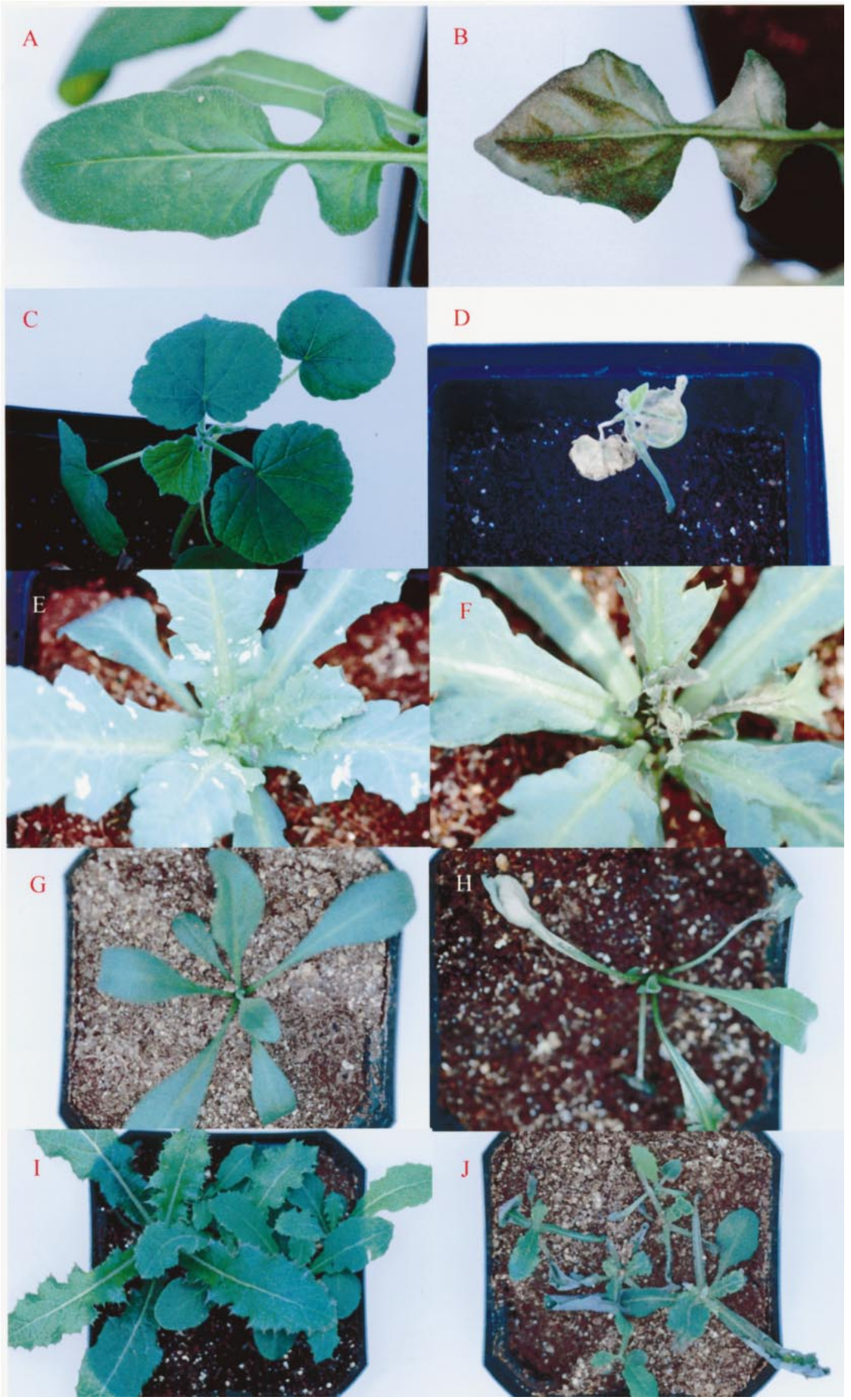
In some cases, if detached leaves were pretreated in an atmosphere of 100 μl L^{-1} ethylene prior to protein treatment, the rate of induced ethylene production was increased (Figure 3). This was true for *P. somniferum, L. esculentum, M. neglecta,* and *C. draba.* Ethylene pretreatment appears to "sensitize" leaf tissue to Nep1 with respect to ethylene biosynthesis. The mechanism of this process is unknown, but the ethylene pretreatment effect has been previously reported for this protein on *E. coca* (Bailey et al. 1997a) and for EIX on *N. tabacum* (Bailey et al. 1995). Except for EIX treated *N. tabacum* (Bailey et al. 1995), induced tissue necrosis did not appear to be affected by the ethylene pretreatment.

The Nep1 protein, applied as a 200-nM spray, caused extensive foliar necrosis to *C. solstitialis, A. theophrasti, C. maculosa,* and *S. oleraceus* (Figure 4). Damage to *P. somniferum* was concentrated on young leaves in the crown of the plant and to the leaf margins. Necrosis was observed within 48 h of treatment, but the treatment did not kill the meristematic tissues. Other microbial polypeptides, e.g., harpin (Bauer et al. 1997; Dong et al. 1997; Qui et al. 1997; Theisen et al. 1997) and xylanase (unpublished), that induce necrosis when infiltrated into sensitive tissue do not cause macroscopic necrosis when applied as a foliar spray. The ability of the Nep1 protein to cause necrosis when applied as a foliar spray raises the possibility of using specific microbial polypeptides as bioherbicides.

By elucidating the signal transduction pathways of plant responses induced by Nep1, we are hoping to better understand the role of proteinaceous elicitors and toxins in plant/pathogen interactions. Perhaps Nep1 is one of the signals perceived by the plant after fungal infection that ultimately contributes to disease development. Recently, Madhosingh (1995a) found that sterile culture filtrates of *F. oxysporum* f.sp. *radicis-lycopersici* were able to induce wilt symptoms in intact *L. esculentum* plants and inhibit the germination of *L. esculentum* seeds. Madhosingh (1995b) also showed that there was a positive correlation between the virulence of various *radicis-lycopersici* isolates and the wilt-inducing capability of their filtrates. Sutherland and Pegg (1995) have purified a toxin fraction from *F. oxysporum* f.sp. *lycopersici* filtrates that separates into two protein bands, 56 and 61 kDa, under denaturing conditions. This toxin fraction caused disease-like symptoms when injected into a *L. esculentum* cultivar that is susceptible to the fungal pathogen, whereas plants of a resistant cultivar did not respond to the toxin. These studies leave open the possibility that there may be a role for Nep1 in plant pathogenesis.

Harpin, a protein isolated from bacterial pathogens in the *Erwinia* genus, has also been shown to contribute to pathogenicity (Bauer et al. 1995), but it can induce hypersensitive-like necrosis in leaves of nonhost species as well (Wei et al. 1992). Proteins of fungal origin have been shown to function as elicitors of plant defense responses too. These include EIX from *T. viride* (Anderson et al. 1993; Bailey et al. 1995) and a group of small molecular weight proteins from *Phytophthora* species known as elicitins (Bonnet et al. 1996; Yu 1995). *Avr*-gene products from *Cladosporium fulvum* have also been well studied (Hammond-Kosack et al. 1996; Kooman-Gersmann et al. 1996; May et al. 1996). It is, therefore, quite possible that Nep1 functions as an elicitor of defense responses rather than as a toxin.

Ultimately, Nep1 or other microbial polypeptides may have value in agriculture. The protein itself or an active oligopeptide derived from the full-length protein could be a biologically based alternative to the currently available synthetic organic herbicides used in weed management. At the current concentration reported here, about 6 g or 250 μmoles ha^{-1} of the purified protein would be needed. This is as active or more active than some of the most effective herbicides in use. We observed that Nep1 affected plants from several genera but not to the same degree. The levels of induced necrosis and ethylene biosynthesis were very species dependent. Alternatively, polypeptides used at relatively low concentrations could activate crop plant defense mechanisms against pathogens in the field (Bauer et al. 1997; Dong et al. 1997; Qui et al. 1997; Theisen et al. 1997); however, one might expect that certain weeds grown in the field would also have their defense systems activated and be more resistance to pests. More research into the effects Nep1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and other peptides on weeds is necessary to determine the usefulness of these compounds in agriculture.

Sources of Materials

[1] Fast Protein Liquid Chromatograph, Pharmica Biotech Inc., 800 Centennial Avenue, Piscataway, NJ, 08855-1327.

[2] Jiffy-Mix Plus, Jiffy Products of America, Inc., 1119 Lyon Road, Batavia, IL 60510-4303.

[3] Hewlett-Packard Co., Analytical Business Center, 2850 Centerville Road, Wilmington, DE 19808-1610.

[4] Silwet L-77, Osi Specialties, Terrytown, NY 10591-6728.

Acknowledgments

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FIGURE 4. Response of 4- to 6-wk-old *Centaurea solstitialis* (A and B), *Abutilon theophrasti* (C and D), *Papaver somniferum* (E and F), *Centaurea maculosa* (G and H), and *Sonchus oleraceus* (I and J) seedlings sprayed with 0.21 μM Nep1 in 0.1% (V/V) Silwet in water at the rate of 120 ml/m^2 (B, D, F, H, and J). Controls (A, C, E, G, and I) were treated with 0.1% Silwet. Plants were placed in a growth chamber with a 12:12-h light:dark cycle at 29 and 21 C, respectively. Photographs were taken 7 d after treatment.

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